Journal of Neuro-Oncology **11**: 157–164, 1991.

Laboratory Investigation

Glutathione levels and chemosensitizing effects of buthionine sulfoximine in human malignant glioma cells

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Key words: glutathione, malignant glioma, buthionine sulfoximine, MTT assay, chemosensitization

Summary

Biopsy samples and cultured cells derived from them were obtained from 39 patients with malignant glioma and were analyzed for 1) glutathione (GSH) content; 2) sensitivity to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and/or nitrogen mustard (HN2) treatment and 3) the effect of buthionine sulfoximine (BSO) treatment on BCNU and/or HN2 cytotoxicity. The average GSH concentration of biopsy specimens was lower than those of cultured cells (2.36 ± 0.44 vs. 11.42 ± 2.32 nmol/10^6 cells). While some of the tumor specimens were sensitive to either BCNU or HN2, the majority were resistant to both. However, 8 of 23 tumors tested showed enhanced sensitivity to BCNU following treatment with BSO. Five of 17 tumors were similarly sensitized to HN2 by BSO. These results suggest that BSO chemosensitization may be of value for certain patients and that screening assays may help identify treatment-sensitive individuals.

Introduction

Malignant glioma is the most common brain tumor and is generally fatal irrespective of conventional treatments. Because of its poor prognosis, aggressive and novel forms of therapy are being sought. Altered fractionation schemes and radiosensitizing drugs [1–3] are in use in an attempt to overcome the inherent radioresistance characteristic of gliomas. As well, a trial of high dose 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) followed by autologous marrow rescue has been undertaken in an attempt to improve long term survival [4].

Studies using experimental tumor models also suggest promising therapeutic strategies. One involves the use of buthionine sulfoximine(BSO) to sensitize tumor cells to the effects of chemotherapeutic agents. Elevated glutathione (GSH) levels have been associated with drug resistance in human tumor cells [5]. BSO is an effective inhibitor of GSH synthesis [6]. By depleting intracellular GSH levels, BSO has been shown, in both *in vitro* and *in vivo* systems, to sensitize neoplastic cells to the effects of certain drugs (reviewed in 7). In many studies, the chemosensitizing effects have been greater for tumors than for dose-limiting normal tissues, resulting in positive therapeutic benefits [7, 8]. Little information is currently available concerning the *in situ* levels of GSH in human malignant gliomas or the role tumor GSH may play in the clinical sensitivity of such tumors to treatment with chemotherapeutic agents. However, recent laboratory studies which have investigated the effects of BSO on the sensitivity of two rat gliomaa cell lines

to treatment with BCNU and nirogen mustard (HN2) suggest that tumor GSH levels may influence response to chemotherapy [10]. In these studies, a small degree of chemosensitization was achieved when glioma cells were treated with BSO and BCNU. However, the combination of BSO and HN2 resulted in significant sensitization in both HN2 resistant and sensitive cells.

We are currently evaluating whether pre-treatment screening assays can be used in conjunction with clinical parameters to identify therapy appropriate for individual patients with malignant glioma. In particular, we wish to determine whether modulating tumor GSH levels will alter the sensitivity of human glioma cells to treatment with BCNU or HN2. In this paper, we report the results of our studies which have measured GSH levels in a series of human malignant glioma biopsy specimens and in cell lines derived from them. In addition, we report on the use of a rapid *in vitro* proliferative assay to screen the effects of GSH-depletion on tumor cell chemosensitivity.

Materials and methods

Biopsies

All procedures were approved in advance by the Institute's Ethics Committee on human experimentation. Written informed consent was obtained from all patients prior to surgery. A portion of the diagnostic biopsy obtained from patients with grade III or IV astrocytoma or glioblastoma was allocated to these studies. Biopsy specimens were placed in sterile saline and maintained on wet ice during transport to the lab. Most specimens were processed 2–5 hours post-surgery. Biopsies were rinsed free of blood, minced with scalpels and then dissociated by continuous agitation for 30 min at 37° C in an enzyme cocktail consisting of collagenase (0.25%) (Gibco), DNAse (0.04%) (Sigma) and Pronase (0.05%) (Calbiochem) in PBS (Gibco) followed by a 30 min holding at 4° C. Cells were washed free of enzymes and known numbers of trypan blue excluding cells were prepared for GSH analysis or chemosensitivity testing. Cells in DMEM/F12 medium supplemented with 10% fetal calf serum (Flow) and 1 mM HEPES buffer (Gibco) were also seeded onto 60 mm tissue culture dishes to establish primary cultures.

GSH analysis

Cell obtained directly from enzymatic dissociation of biopsy specimens or from cultured cells were assayed for intracellular GSH content using a biochemical assay [11].

Chemosensitivity testing

Enzymatically dissociated biopsy material or early passage cell lines derived from these biopsies were assayed for sensitivity to BCNU or HN2 using a modification of the MTT proliferation assay originally described by Mosmann [12]. In these studies, cells (5×10^4 to 1×10^5/well) in complete DMEM/F12 culture medium were seeded into 96 well plates and 48 hr later, BSO (Sigma) (0.1 mM) was added to selected wells. After a 24 hr incubation, BSO was removed and graded concentrations of freshly prepared BCNU (20–100 μM) or HN2 (1–6 μM) were added for 2 hr. Following removal of the drugs, fresh medium was added and plates were incubated for an additional 7 days at 37° C. MTT-formazan crystals were solubilized with acid isopropanol (0.04N HCl in isopropanol) and the plates were read by a Dynatech MR 600 Microplate reader at test and reference wavelengths of 570 and 630 nm, respectively. The absorbance was used as an index of the relative numbers of proliferating cells in control and drug-treated groups. Reduction of cell proliferation by 40% or more was used as evidence of tumor cell sensitivity [13].

GSH depletion studies

Cells were seeded onto 60 mm tissue culture dishes and incubated at 37° C for 48 hr. BSO was then

Table 1. Tumor GSH content and sensitivity to treatment with BCNU or HN2 in a series of patients with malignant glioma. The age and sex of each patient at the time of surgery is indicated

Patient	Age/sex	GSH[1] (Biopsy-culture)		BCNU[2] (Control/+BSO)		HN2[2] (Control/+BSO)		BSO[3] (% control)
1	13 F	–	2.58	–	–	–	–	
2	58 M	–	6.04	100	100	87	48	92
3	55 M	–	4.48	96	100	76	73	100
4	51 M	–	4.76	64	93	39	84	21
5	67 M	0.87	6.42	51	28	89	54	82
6	47 M	–	4.56	39	2	81	93	87
7	49 M	0.86	14.3	79	96	77	76	88
8	66 M	0.16	8.46	30	15	87	61	100
9	47 M	–	25.6	30	100	–	–	15
10	53 M	1.18	–	–	–	–	–	
11	63 M	–	7.83	75	63	100	55	93
12	54 M	2.52	46.6	–	–	–	–	
13	40 M	0.13	7.0	64	94*	73	95*	76
14	29 M	0.38	7.24	73	68*	61	100	78
15	67 M	0.42	–	37	24*	–	–	100
16	70 M	2.82	–	–	–	–	–	
17	51 M	0.83	21.3	27	9	74	70	100
18	35 M	5.72	–	–	–	–	–	
19	30 F	2.39	–	68	42*	88	79*	100
20	25 M	2.40	6.13	87	100	84	83	88
21	30 M	1.01	–	–	–	–	–	
22	62 M	2.72	–	81	81	–	–	24
23	66 M	6.60	–	–	–	–	–	
24	8 F	0.66	–	–	–	–	–	
25	28 M	1.19	14.8	48	33*	60	36*	87
26	13 F	6.46	–	–	–	–	–	
27	38 M	4.69	–	–	–	–	–	
28	65 F	0.22	–	–	–	–	–	
29	74 M	2.91	–	50	54*	76	79*	69
30	69 F	1.96	–	76	73	–	–	100
31	46 M	1.61	–	32	23*	–	–	51
32	37 M	11.13	2.97	60	59	94	72	97
33	50 M	2.96	–	–	–	–	–	
34	32 M	5.16	–	–	–	–	–	
35	39 M	0.03	–	–	–	–	–	
36	23 F	0.68	6.3	–	–	–	–	
37	58 M	3.19	15.0	41	49	57	38	86
38	56 M	0.71	–	–	–	–	–	
39	42 M	0.33	16.2	57	73*	–	–	64

[1] The GSH content of the tumor biopsies or the cultured cells is expressed as nmol/10^6 cells.

[2] The cytotoxic effects of BCNU or HN2 treatment are shown for control (drug only) and BSO pretreated cultures. The relative absorbance of control cultures (medium only or medium plus BSO only) was normalized to 100%. The effects of treatment with maximum doses of drug alone or drug + BSO are expressed as a percent of the relevant control culture. Drug sensitivity was defined as $>40\%$ reduction in relative absorbance.

[3] The effect of treatment with BSO only on cell proliferation is expressed as a percentage of the cell proliferation observed in control (medium only) cultures.

* = drug test done with freshly dissociated tumor specimens.

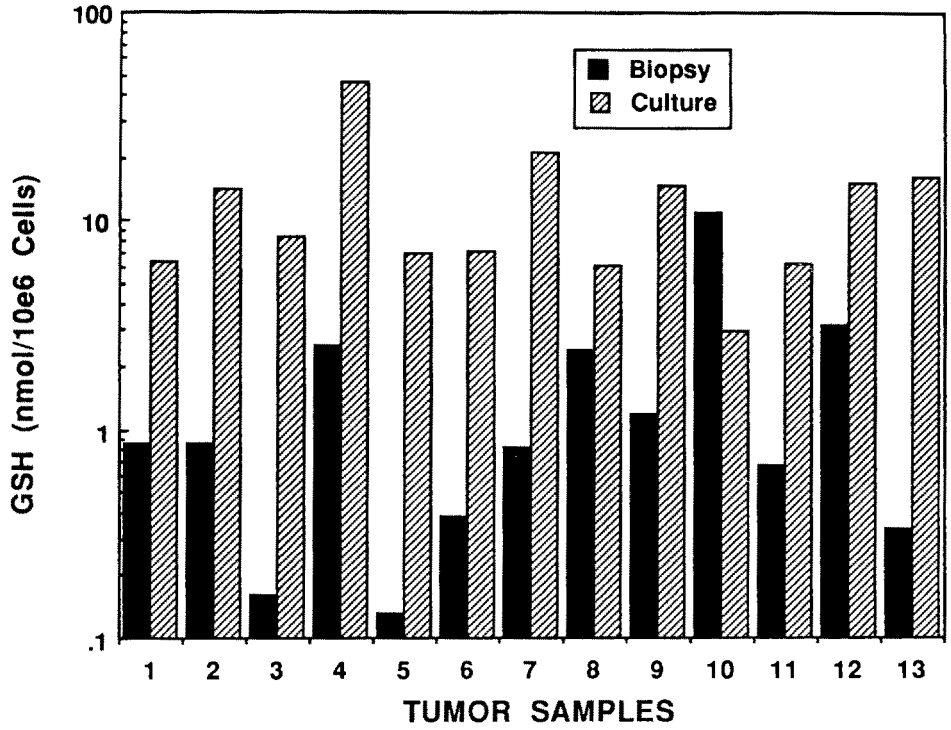

Fig. 1. Comparison of tumor GSH levels in cells derived directly from enzymatic dissociation of biopsy specimens (solid bars) or from the cultured cells derived from these biopsies. Results from 13 individual patients are shown.

added for an additional 24 hr. At the completion of drug exposure, the plates were rinsed with ice cold PBS and cells were prepared for GSH analysis.

Statistics

A nonparametric paired sample test (sign test) was used to evaluate the association between various patient characteristics (e.g. age, sex, tumor GSH) and chemosensitivity.

Results

Patient characteristics

Biopsy material has been obtained from 39 patients with malignant glioma. The age at time of surgery and sex of each patient is listed in Table 1. The GSH content of both the tumor biopsy specimens and the tumor cell lines derived from these biopsies and the results of the chemosensitivity testing are also shown.

Tumor GSH

The intracellular GSH concentrations were determined for both fresh biopsy specimens and cultured cells (Table 1). The mean (± SEM) GSH concentration of biopsy-derived cells was 2.36 ± 0.44 nmol/10^6 cells (range = 0.03 to 11.13 nmol/10^6 cells). GSH values of cultured cells were elevated compared to fresh tumor specimens, and had a mean (± SEM) value of 11.42 ± 2.32 nmol/10^6 cells (range = 2.58 to 46.6). GSH measurements of replicate samples of individual tumors were within 10% of each other. For 13 patients, the GSH content of the biopsy and corresponding cell line was directly compared (Fig. 1). In 12 of 13 cases, the GSH content of cultured tumor cells was increased 2 to 22 fold relative to that of fresh tumor specimens. In the remaining case, the GSH content of

the biopsy specimen was approximately 4-fold greater than that of the corresponding cell line.

Chemosensitivity testing

The effect of BSO on the chemosensitivity of cells treated with BCNU of HN2 is summarized in Table 1. In 9 cases, the biopsy specimens were of sufficient size to allow drug testing to be done with freshly dissociated tumor material. In the remaining 14 cases, biopsy-derived early passage cell lines were used for the chemosensitivity assay. For all cases, the effect of drug ± BSO treatment on cell proliferation was defined as a percent of the control (medium only or medium plus BSO only) absorbance values. For most tumor samples (18 of 23) BSO treatment alone had minimal effects on cell proliferation. When expressed as a percentage of control cell (medium only) proliferation, the cell proliferation in cultures treated with BSO only had a mean value of 79 ± 27% (mean ± SD; range = 15–100%) (Table 1). In six tumors, BSO inhibited cell proliferation by more than 25%. Four categories of tumor response to drug ± BSO treatment were observed: A. resistant to chemical agent both with and without BSO treatment; B. resistant to chemical agent without BSO but sensitive if cells received BSO treatment; C. sensitive to chemical agent and resistant to further sensitization by BSO; D. sensitive to chemical agent and further sensitized by BSO. The majority of samples were resistant to both BCNU and HN2 (12 of 23 and 15 of 17 for BCNU and HN2 respectively). One biopsy was identified as sensitive to both BCNU and HN2. The effects of BSO treatment on chemosensitivity was also highly variable, did not correlate with initial GSH content and was independent of the cytotoxic effects of treatment with BSO alone. Enhanced drug cytotoxicity was observed in 8 of 23 tumors treated with BSO followed by BCNU and in 5 of 17 tumors treated with BSO followed by HN2.

Effect of BSO of GSH content

The effect of a 24 hr exposure to BSO (100 μM) on the GSH content of seven cultured cell lines was determined. In five cases, the GSH concentrations in treated cells were less than 10% of control samples incubated without BSO (i.e. 9, 6, 3, 4 and 1%). In two cases, the GSH levels were below the limits of detection (i.e. 0%).

Discussion

GSH depletion sensitizes human tumor cells to a wide variety of chemotherapeutic agents [14–16]. Although drug resistance in glioma patients is likely to be a multifactorial phenomenon, manipulation of tumor GSH may benefit selected patients. To investigate this possibility, we measured both tumor GSH levels and sensitivity to treatment with BCNU or HN2 alone, or in combination with BSO. A wide range of GSH values was obtained for both the biopsy samples and the cultured cells derived from them. Little comparative information is available concerning the *in situ* GSH values in other populations of brain tumor patients. However, in their analysis of a series of head and neck biopsies, Guichard and colleagues noted a 34-fold variation in GSH content [17]. In our study 28 of 32 samples (87%) were within a 30-fold range of variation. Diurnal variation in GSH levels has been observed in experimental animals [18]. As well, differences in feeding rhythm and the type of diet consumed have been shown to affect tissue GSH content [18]. Regional differences in tumor GSH also have been described for multiple biopsy samples taken from the same tumor [19]. All of these factors could have contributed to the variation in biopsy GSH values reported in this study and that of Guichard *et al.* [17]. Guichard *et al.* noted an 18-fold variation in the GSH content of human tumor cell lines cultured *in vitro*. The glioma cell lines reported in this study also vary in GSH content by a factor of 18. When tumor cells obtained from our biopsies were cultured *in vitro,* the intracellular GSH concentration was increased. This difference in GSH values between the biopsy samples and the cultured cells

was similar to that observed by one of us for human tumor cells grown both as cell lines and as xenografts [19] and may reflect in part, differences in the oxidative stress encountered by cells growing *in vivo* and *in vitro*. The lower biopsy values are unlikely to have been the result of loss of GSH during the post-surgical storage and transport interval since previous studies have shown that in comparison to freshly excised human tumor cells processed without a holding period, no significant loss of tumor biopsy GSH occurs in tumor cells held at 4° C for 4–6 hr prior to analysis [19].

The effect of GSH depletion on the chemosensitivity of tumor cells derived from 23 individual patients has also been determined. These studies are part of a project designed to evaluate the use of predictive assays in identifying treatment-sensitive malignant glioma patients. The MTT assay was chosen as a rapid screening method of proliferative capacity for both the biopsy and cultured cells. Previous studies have established the validity of using proliferative assays as monitors of brain tumor cell drug sensitivities [20, 21]. Both fresh biopsy material and cells which had been expanded by culture *in vitro* were used in the chemosensitivity testing. This latter procedure was necessary to obtain data because frequently the biopsy specimens were small (< 100 mg) and did not yield sufficient numbers of cells for drug testing. Studies by others have demonstrated that the sensitivity of human brain tumor specimens to treatment with BCNU is similar regardless of whether cells are treated immediately after enzyme dissociation from the biopsy or whether they are treated after a few passages *in vitro* [22]. We obtained similar results when freshly dissociated biopsy cells and the corresponding early passage cultured cells were compared in terms of sensitivity to BCNU or HN2.

The majority of the samples tested, either as biopsy material or as cultured cells, were found to be resistant to BCNU. These results are in agreement with the previous work by Scudiero *et al.* [23] who have determined the sensitivity to nitrosoureas for a series of human tumor cell lines. The majority (70%) of the cell lines studied [23 and Day unpublished] were found to be nitrosourea resistant. This resistance reflected the ability of those cells to repair 06-methylguanine lesions in their DNA efficiently. Such repair proficient cells are defined as having the Mer+ phenotype. Continuous cell lines produced from the biopsies obtained in this study are currently being analyzed for Mer phenotype. Initial results have identified tumor cell lines from three patients as being repair deficient, or Mer−. The two of these patients were identified as being BCNU sensitive in the initial drug sensitivity screen (i.e. had a greater than 40% decrease in cell proliferation). The third patient had an intermediate sensitivity to BCNU (21% reduction in cell proliferation), but according to the criteria adopted for this study, remains classified as a resistant patient. A majority of the patients tested were also resistant to HN2, an agent whose mode of cytotoxicity does not involve the production of 06-methylguanine lesions [24].

Eight of 23 tumor specimens exhibited enhanced sensitization to BCNU following treatment with BSO. Of these, seven were sensitive and one was resistant to BCNU in the absence of BSO. Five tumor samples exhibited enhanced sensitivity to HN2 following BSO treatment. All but one were initially resistant to HN2. The reasons why BSO failed to produce chemosensitization in all tumors remain to be defined. However, other investigators have reported a similar lack of enhanced tumor cell kill following BSO [25–27]. In addition inconsistent results have been reported regarding BSO's effect on the chemosensitivity of a single cell line treated with a variety of drugs as well as it's effect on different tumor types treated with a single agent [reviewed in 25]. In this and in previous studies, insufficient GSH depletion is unlikely to have been the causative factor [25–27]. In order to standardize our chemosensitivity screening procedure, a fixed dose of BSO was used for all samples. When tested with cultured cell lines derived from seven biopsies, this dose of BSO reduced GSH concentrations to < 10% of control values over a 24 hr period. Of these seven tumor samples, four continued to be resistant to HN2 despite depletion of GSH to 6, 4, 1 and 0% of initial values. Sensitization by treatment with BSO was also found to be independent of whether treatment with BSO alone was cytotoxic. The role of GSH rebound synthesis

following BSO treatment can be considered as a possible explanation for the lack of chemosensitization in some tumors. In the case of BCNU, the production of DNA interstrand cross-links requires 6–12 hrs for completion [28]. Any significant increase in GSH levels during this time could theoretically reduce cytotoxicity by quenching the formation of cross-links [29]. While this possibility cannot be excluded for the *in vitro* studies presented here, experiments with *in vivo* tumor models would argue against this as being a major deterrent to the possible clinical use of BSO as the recovery to control GSH values of three different murine tumors treated *in vivo* with BSO was shown to require approximately 48 hr [30].

At the present time, no significant correlation between intial tumor GSH level, Mer phenotype, tumor histology, patient age or sex, or use of biopsy or cultured cells is evident to explain the enhanced chemosensitivity observed with selected bipsies. This lack of correlation may reflect the small numbers of patients accrued to date. Alternatively, some combination of factors other than those defined above may be critical in determining the sensitivity of a given tumor to BSO's chemosensitizing effects. Nonetheless, these results suggest that BSO treatment may be of value in selected patients and that preliminary screening of the chemotherapy and BSO sensitivities of individual patients may help to identify treatment sensitive individuals.

Acknowledgements

Supported by awards from the Alberta Cancer Board Research Initiative Program, the National Cancer Institute of Canada and an Alberta Heritage Foundation for Medical Research Establishment/Scholarship grant to R. Day. The authors wish to thank Gina Kennedy and Linda Wilson for typing the manuscript and Richard Besse for preparing the figures.

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